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54 Title: PEPTIDE WITH RADIO-PROTECTIVE EFFECT

57 Abstract

The invention concerns a peptide with radio protective effect that comprises a modified form and/or, optionally, a modified fragment of the Bowman-Birk-Protease-Inhibitors (BBI).

Peptide with radio-protective effect

The present invention relates to a peptide with radio- protective effect.

A peptide of this kind is for example known from the publication by Dittman, K., et al., "Bowman-Birk proteinase inhibitor (BBI) modulates radiosensitivity and radiation-induced differentiation of human fibroblasts in culture," Radiotherapy and Oncology 34, pages 137-143 (1995).

The radio-protective effect of a peptide is understood to be its protective activity for cells, tissues or organisms against damaging radiation. For living organisms, particularly damaging radiation is ionizing radiation and UV radiation, that is, energy-rich types of radiation. A peptide displays a radio-protective effect when the damage caused by the radiation is reduced by this peptide. The mechanisms underlying a radio- protective effect are at present still completely unexplained.

Damage caused by energy-rich radiation is for example the alteration of DNA, or mutagenesis, which can lead to the formation of tumors, but also the degeneration, atrophy, fibrosing or necrosing of tissues that are subjected to high radiation.

Thus for example the formation of malignant melanoma is promoted by high exposure of the skin to the sun.

The human organism is confronted with especially high radiation intensities during high exposure to sunlight, but also during x-ray diagnostics or radiation treatment of tumor diseases.

Protection against UV radiation is offered, for example, by UV-filtering substances such as are contained in suntan lotion. As a protection against ionizing radiation, the shielding of parts of the body not to be irradiated and the most precise possible local application of the radiation are used.

It has been recognized very recently that there are peptides that can exhibit a radio-protective effect. A peptide of this kind is the initially-mentioned Bowman-Birk Protease Inhibitor (BBI), a long-known inhibitor of the serine proteases trypsin and chymotrypsin, that is contained in large amounts in soybeans.

The amino acid sequence of the BBI is known, and the corresponding gene of the soybean was already cloned in 1984 (Hammond, R.W., "Molecular Cloning and Analysis of a Gene Coding for the Bowman-Birk Protease Inhibitor in Soybean," J. Biol. Chem. 269, pages 9883-9890 (1984)).

The BBI includes 71 amino acids and has a molecular weight of about 8000 Daltons. One of the characteristics of the BBI is the large number of fourteen cysteine residues, that form seven disulfide bridges and thus participate in an essential manner in the folding or secondary structure of the BBI.

By chemical and enzymatic cleavage with cyanogen bromide (CNBr) and the protease pepsin, the BBI is split into two halves, of which one displays trypsin-inhibiting activity and the other chymotrypsin-inhibiting activity (Odani, S., and Ikenaka, T., "Studies on Soybean Trypsin Inhibitors," J. Biochem. 83, pages 747-753 (1978)).

Besides the protease-inhibitor function, two additional physiological activities of the BBI were demonstrated, namely an anticarcinogenic activity and the radio-protective activity already mentioned.

In the publication by Clair, B.H.St., "Suppression of Dimethylhydrazine-induced Carcinogenesis in Mice by Dietary Addition of the Bowman-Birk Protease Inhibitor," Cancer Research 50, pages 580-586 (1990), it was shown that the BBI has an anticarcinogenic effect. In in vitro experiments with cultured cells it was shown that the chymotrypsin-inhibiting domain could prevent a malignant transformation of the cells. In in vivo experiments, in which tumors were induced in mice by carcinogens and the BBI was administered orally, it was shown, however, that the trypsin-inhibiting domain of the BBI is necessary for the suppression of the tumor formation. This anticarcinogenic effect is thus directly traced back to the protease-inhibiting effect of the BBI. It was further shown in the in vivo experiments that autoclaved, i.e. heat-denatured BBI could no longer exhibit any anticarcinogenic effect.

The radio-protective effect of the BBI already mentioned was described in the publication by Dittmann et al. (1995) cited initially. Here it could be shown that the BBI reduced the radiation-induced dying-off of cultured human fibroblasts. Fibroblasts are connective tissue cells that are present for example in large quantities in the skin.

In US 5,376,373, a method was proposed in which radiation-induced weight and hair loss is inhibited by oral administration of a "concentrate" obtained from soybeans, in which the BBI is contained. In the isolation of the BBI-concentrate, a soybean extract is repeatedly fragmented, precipitated, ultrafiltered, diluted and again concentrated to obtain the radio-protective product. What additional components, for example other protease inhibitors or the like, are contained in the concentrate is not known.

A problem on oral intake of BBI with the diet consists in the fact that it was shown in rats that large amounts of trypsin inhibitors lead to hypertrophy and hyperplasia of pancreatic cells and also to weight loss. In rats that took in trypsin inhibitors from soybeans with their feed for long periods of time, pancreatic tumors developed.

Another problem in the administration of serine protease inhibitors, especially with intravenous administration, consists in the fact that blood clotting, in which the serine proteases are heavily involved, can be disturbed.

With this background, it is the problem of the present invention to prepare a peptide with radio-protective effect that can be produced with low expenditure and in which the disadvantages of the radio-protective products already known are avoided.

This problem is solved according to the invention by a peptide with radio-protective effect including a modified form and/or a possibly modified fragment of the Bowman-Birk protease inhibitor.

The Inventor has surprisingly established that structurally modified forms and even fragments of the BBI display a radio-protective effect. It is thus no longer necessary to prepare the BBI in its original form in order to obtain a peptide with the desired radio-protective effect.

Modified, in the sense of the invention, is understood to mean any change in the structure or conformation of the BBI as well as any change in its amino acid sequence, whether due to chemical or enzymatic addition or to removal of individual groups of amino acids or due to the exchange of individual amino acids. A modified form of the BBI also contains a peptide in which additional amino acids were added to the N– or C-terminal ends of the BBI, for example domains of additional proteins or peptides that make the purification of the BBI easier and/or further strengthen its radio-protective effect.

A fragment in the sense of the invention is understood to be any fragment of the BBI in which either only single amino acids or longer sections of amino acids of the BBI are missing. These kinds of fragments encompass for example individual domains of the BBI. According to the invention, such fragments can also be modified in the sense direction shown above.

Modified forms of the BBI or BBI fragments can be produced either by treatment of the original and thus unaltered BBI with chemicals or enzymes or by synthesis with chemical or molecular biological methods.

It was not to be anticipated that modified forms or fragments of the BBI can also display a radio-protective effect, since usually modifications, especially when they involve the conformation of the peptide, destroy or at least greatly reduce the physiological activities of a peptide.

In addition, it had been shown for the anticarcinogenic activity of the BBI that its anticarcinogenic effect completely disappeared due to impairment of the BBI structure by heat denaturing.

Modified forms or fragments of the BBI can be handled without problem because both during their production and on storage no attention has to be paid as to whether a contact with modifying agents, proteases or the like, is excluded.

In this way, the problem underlying the invention is completely solved.

In a preferred embodiment, the new peptide displays no protease-inhibiting effect against trypsin and chymotrypsin.

A peptide of this type has the considerable advantage, besides the radio-protective effect, of not simultaneously also blocking the proteases trypsin or chymotrypsin. This is particularly advantageous when the peptide is to be used to protect cells, tissues or organisms from radiation since, as already explained above, at least peptides with trypsin-inhibiting effect are damaging to pancreatic cells and can even lead to tumor formation.

The fact that modified forms of the BBI are radio-protectively active, without at the same time having a protease-inhibiting activity against trypsin and chymotrypsin, was all the more astonishing since it had been assumed up to now that for the additional activity of the BBI, i.e. its anticarcinogenic effect, one of these protease-inhibiting domains is necessary (Clair et al., 1990).

A peptide in accordance with the invention has a single well-defined effect, that is, protection from radiation. If it is used in the presence of trypsin and chymotrypsin, these serine proteases are not simultaneously hindered in their activity. Thus the above-cited pancreas-damaging side effects are avoided.

In another preferred embodiment, the peptide has at least two cysteine residues that are present in reduced form

This measure has the advantage that a peptide of this type, even in the presence of reducing agents, can be used as a radio-protective agent. With peptides that contain cysteine residues, reducing agents lead to their sulfur residues being protonated and thus existing as SH groups. Under oxidizing conditions, the SH groups form disulfide bridges between each other that substantially influence the folding of the peptide.

Reducing [typo in original] agents are often used in the purification of proteins and peptides. Under physiological conditions, reducing conditions exist inside the cells because of the presence of glutathione.

The preparation of a peptide, in which the cysteine residues are present in reduced form, has the further advantage that it can be produced without difficulty using molecular biological methods in bacteria. Such expression in bacteria is much simpler and more efficient than expression in yeasts and higher eukaryotic cells and permits especially high yields. For proteins or peptides, however, in which the cysteine residues are bound to each other covalently over disulfide bridges, this system is unsuitable since disulfide bridges cannot be formed in bacteria. A reduced form of the BBI, on the other hand, can be readily expressed in bacteria.

In another preferred embodiment, at least some of the amino acid residues are present in alkylated form.

By alkylation is understood the modification of individual amino acids with alkyl groups, usually methyl groups This modification occurs using the so-called alkylation reagents, for example iodoacetamide.

By treating the BBI with iodoacetamide, after reduction of the disulfide bridges, the SH groups of the cysteine are provided with a methyl group, that is, alkylated. Re-oxidation of the SH groups is then no longer possible. Thus even under oxidative conditions the re-formation of the disulfide bridges is prevented.

Alkylation of the BBI or of fragments of the BBI has the advantage of obtaining cysteine residues in their reduced form, and thus stabilized.

In an another preferred embodiment, the peptide has less than 20, particularly preferably less than ten amino acids.

Such a drastically diminished BBI fragment has the considerable advantage that when it is used on cells, tissues or in the entire organism, because of its small size, it is distributed and penetrates much better and faster than the whole BBI containing over 70 amino acids.

Good distribution and easier penetration into the tissue is important in the use of the peptide as a radio-protector, since it can then exhibit its protective effect against radiation rapidly and comprehensively.

In another preferred embodiment, the peptide is a nonapeptide and displays the sequence SEQ ID No.: 1 from the attached Sequence Protocol.

As is to be inferred from the embodiment examples, the inventor has succeeded in demonstrating that this nonapeptide exerts just as great a radio-protective effect on human fibroblasts as the whole BBI. The use of a peptide that is very greatly diminished relative to the whole BBI, and that includes only nine amino acids (nonapeptide), has the considerable advantage that this nonapeptide can be prepared more easily. That is, a nonapeptide can be produced without difficulty by chemical synthesis, also called the Merrifield synthesis. This type of peptide synthesis is a widespread and well-established synthetic method by means of which this kind of peptide can be obtained in large amounts in high purity.

In another preferred embodiment, the peptide is a nonapeptide with the sequence SEQ ID No.: 2 from the attached Sequence Protocol.

In this nonapeptide, the sequence taken from the naturally-occurring BBI is modified by one amino acid. The serine from the sequence SEQ ID NO.: 1, i.e. a hydrophilic amino acid, is exchanged in the SEQ ID No.: 2 for a valine, i.e. a hydrophobic amino acid. A radio-protective effect could also be demonstrated for this modified peptide, as can be seen from the embodiment examples. This modified nonapeptide with the sequence SEQ ID NO.: 2 can also be synthesized without difficulty by the Merrifield synthesis.

In another preferred embodiment, the terminal cysteine residues of the peptides with the sequences SEQ ID NO.: 1 and 2 are bonded covalently to each other.

In this way, ring-form or cyclic peptides result that display high stability in an oxidizing environment. An oxidizing environment is present for example in the extracellular matrix of the connective tissue and in all parts of the body that have contact with the outside air.

Since both the linear and the cyclic peptides with the sequences SEQ ID No.: 1 and 2 display a radio-protective effect, they are effective both in reducing and also in oxidizing environments as radiation protection and can thus be used universally.

In another preferred embodiment, the peptide displays the sequence SEQ ID No.: 3 from the attached Sequence Protocol.

This peptide includes only seven amino acids, and is thus a heptapeptide. Due to the further shortening of the peptide, with retention of its radio-protective effect, the peptide is to be produced still more rapidly and cheaply and is more easily applied.

All peptides with the sequences SEQ ID No.: 1-3, whether they are cyclic or linear, have the considerable advantage of possessing no protease-inhibiting activity. They can thus be used without the undesired side effect of blocking the digestive enzymes trypsin and chymotrypsin and other serine proteases.

In another preferred embodiment, at least one of the amino acids of the peptide displays a protective group.

These kinds of protective groups can be any of the protective groups known in peptide chemistry, it being preferable that the C-terminal amino acid display an acetyl group and/or the N-terminal amino acid display an amide group.

These protective groups have the advantage of protecting the peptide from the attack of exopeptidases, so that the peptides have a much higher stability in a biological environment, such as for example in cell culture or in the organism. Protective groups that block the C-terminal carboxyl and the N-terminal amino groups of peptides, such as the acetyl and amide groups referred to, also provide that the peptides do not enter into additional peptide bonds with other peptides or between each other, so that tandemization of the peptides is also reliably prevented. With several peptides linked together it can no longer be reliably assumed that the radio-protective effect is retained.

Accordingly, the protective groups also ensure that the peptides remain in their radio-protectively effective structure

Another aspect of the present invention is the use of one or more of the peptides mentioned as radio-protective agents.

This use as radio-protective agents includes any use in which the peptide is used for protection against radiation, whether ionizing radiation, UV radiation, or electromagnetic radiation.

Especially preferably, a peptide in accordance with the invention is used for protection against ionizing radiation, in particular of normal tissue in the radiation treatment of tumor patients.

In this type of radiation treatment, ionizing radiation is in fact used for the treatment of most malignant tumors. In the course of this, the tumor tissue should be maximally affected and at the same time the surrounding healthy normal tissue should be maximally spared.

In order to keep the exposure of the normal tissue as low as possible, the radiation is if possible applied in a localized manner. However, the protection of the normal tissue lying in the direct vicinity of the tumor tissue is very problematic. A peptide in accordance with the invention can be used ideally here since it can be used either locally or generally, e.g. by way of the bloodstream. Particularly with the small peptides with the sequences SEQ ID No.: 1-3, rapid and uniform distribution in the tissues is achieved, and thus also rapid protective efficacy against the radiation. Moreover, all the peptides in accordance with the invention display, in both oxidative and reducing environments, high stability and at the same time radio-protective efficacy, which additionally favors their therapeutic usefulness.

Preferably, a peptide in accordance with the invention is also used against UV radiation, especially against the UV radiation in sunlight.

For this, it is advantageous that even under oxidative conditions, for example in the air, the peptides are very stable and can exert a long-lasting protective effect against UV radiation. They are therefore suitable for use as skin protection against high solar irradiation. The use of the nona- and heptapeptides in accordance with the invention also has the advantage that because of their small size the peptides can penetrate into the skin and are stable there for a long time.

The invention relates in addition to a pharmaceutical composition, in particular for intravenous administration, that contains one or more of the peptides in accordance with the invention in a radio-protectively effective quantity.

For this purpose, the peptides can be prepared in the respective appropriate, common pharmaceutical forms. Besides intravenous administration, percutaneous administration or local injection into, for example, regions of the body or body cavities directly affected by radiation can be considered.

In a pharmaceutical composition of this kind, it is advantageous that the peptides exhibit their radio-protective effect without at the same time triggering threatening immune reactions. Because of their small size, the peptides in accordance with the invention have in fact only low immunogenicity, so that under normal conditions when the pharmaceutical composition is used on the human or animal body no allergic reactions are to be expected, and the peptides also are not eliminated from the respective organism through the agency of antibodies.

The invention also relates to a cosmetic composition for application on to the skin, which is characterized by the fact that it contains one or more of the peptides in accordance with the invention in a radio-protectively efficacious amount.

A cosmetic composition of this kind can for example be presented as suntan lotion, skin cream or the like, and then contains the usual ingredients of these kinds of compositions such as oils, emulsions, pigments, etc. It is to be understood that the cosmetic composition can also contain in addition UV filters such as derivatives of p-aminobenzoic acid, salicylic acid, cinnamic acid, dibenzoylmethane or the like.

Due to the radio-protective efficacy of the peptides in accordance with the invention, such a cosmetic composition offers ideal protection, especially against the UV radiation of sunlight. Since the peptides in accordance with the invention, because of their small size, even penetrate into the skin and are therefore stable for a long time, a long-term protection against radiation can be achieved.

The invention further relates to a nucleic acid that has a sequence section coding for a peptide in accordance with the invention and possibly control sequences necessary for the expression of the nucleic acid.

A nucleic acid of this kind is advantageously used for the preparation of a peptide in accordance with the invention using techniques of molecular biology, for which it is preferably contained in an expression vector.

The preparation of a peptide in accordance with the invention by nucleic acid expression has the advantage of being an especially simple possibility for producing the peptide in practically unlimited quantities and for modifying it in simple ways while the corresponding coding sequence is modified at the nucleic acid level. A number of standard methods such as in vitro mutagenesis, site-directed mutagenesis, oligonucleotide synthesis, PCR, etc., are known for this.

As an expression system, either an in vitro expression system, for example a reticulocyte lysate, or an in vivo expression in bacteria, yeasts, or eukaryotic cells can be used, with the appropriate expression vectors being applied in each case. Since for the radio-protective effect of the peptides in accordance with the invention the formation of disulfide bridges is not absolutely necessary, the expression can occur in bacteria, in which disulfide bridges are not formed.

To make the production and purification of the peptides in accordance with the invention easier, they can also be synthesized as fusion peptides, which means that, for amino acid sections or domains of known proteins, coding sequences are fused on to the nucleic acids in accordance with the invention, whereby on expression a continuous peptide is produced. Examples of such fused-on amino acid sections are for example so-called histidine tags, through which expressed fusion proteins can be purified by way of nickel chelate columns, or antigen determinants, which permit the peptides to be purified over suitable antibody affinity columns.

In an alternative preferred method for the production of a peptide in accordance with the invention, the unmodified BBI is cleaved proteolytically and/or chemically.

In this method, a BBI purified from soybeans (seeds) or BBI produced by molecular biology can be used as the starting material, and the radio-protectively efficacious modified forms or fragments of the BBI are then obtained from it with (bio)chemical methods. In this way, for example, proteases can be used that cleave the BBI, for example pepsin, and/or chemical cleavages by means of cyanogen bromide.

It is to be understood that the previously mentioned characteristics and those to be explained below can be used not only in the respective combinations given, but also in other combinations or alone, without departing from the scope of the present invention.

Further advantages appear from the following embodiment examples and in connection with the drawing, in which

Fig. 1 shows in the form of a bar chart the result of a so-called clonogenic assay with unmodified and modified BBI

Fig. 2 shows as a bar chart the chymotrypsin-inhibiting activity of unmodified and modified BBI

Fig. 3 shows the elution profile of a chromatographic separation of BBI and BBI fragments

Fig. 4 shows as a bar chart the result of a clonogenic assay with selected fractions from the chromatography in Fig. 3

Fig. 5 shows as a bar chart the result of a clonogenic assay with BBI and four peptides in accordance with the invention under ionizing radiation;

Fig. 6 shows as a bar chart the result of a clonogenic assay with BBI and four peptides in accordance with the invention under UV irradiation;

Fig. 7 shows as a bar chart the chymotrypsin-inhibiting activity of BBI and four peptides in accordance with the invention; and

Fig. 8 shows as a bar chart the trypsin-inhibiting activity of BBI and four peptides in accordance with the invention.

Example 1. Radio-protective effect and protease-inhibiting activity of BBI and modified BBI forms

A. Radio-protective effect

The study of the radio-protective effect of BBI and modified forms of BBI was carried out in the so-called clonogenic assay, which is described for example in Dittmann, K., et al., Radiotherapy and Oncology 34, pages 137-143 (1995), and which is briefly illustrated below.

1.1. Clonogenic assay

Normal human fibroblasts were cultured in Dulbecco's Modified Eagles Medium (DMEM) with 10% fetal calf serum under standard conditions. At each cell passage, the cell count was determined and in the application of subcultures the cells were seeded using a density of 2×10^4 per cm^2.

For the clonogenic assay, secondary fibroblasts were trypsinized with 0.05% trypsin and 0.1% EDTA and seeded with a cell density of 50 cells per cm^2 in 6-well tissue culture plates. The cells were cultured with 2 ml DMEM with 20% fetal calf serum per depression (well).

After 24 hours, the medium was removed and the cells were incubated for 16 hours either in additive-free control medium ("O" in the figures) or in medium that in each case contained 10 µM of the BBI or a modified BBI form. The nona- and heptapeptides in accordance with the invention (see Example 3) were introduced at a concentration of 80 µM.

After this followed irradiation with ionizing radiation, with the energy dose being either 2 or 4 Gray (Gy). This energy dose corresponds to the total amount of radiation energy applied in the mass unit Joule/kg (= Gray). A 6 MeV linear accelerator (Mevatron, Siemens) was used for the irradiation.

The cells were then cultured in BBI-free culture medium for eight more days in order to permit colony formation

The cells were then fixed, stained and counted, with the absolute number of clones counted being given by "K" in the figures.

A clone is thus a colony or a cell cluster that arises due to division of a cell within the 8-day culturing. The number of clones corresponds to the extent to which the human fibroblasts have survived the irradiation. Therefore, in what follows, reference will also be made to "clonogenic survival" of the cells. When many cells die during the irradiation of the cells, then after eight days only a few clones are formed; if many cells survive, then many clones can be counted after 8 days of culture. Thus the clonogenic survival of the cells after irradiation is a direct measure of the radio-protective effect of the BBI product used.

1.2. Radio-protective effect of BBI and modified BBI forms

In the clonogenic assay, the results of which are shown in Fig. 1, four different preparations were tested: in the control preparation indicated by "O", no BBI was added. In the preparation indicated by "BBI," whole BBI (from the Sigma Biochemicals company) was used, and in the preparation indicated by "BBI-R," the whole BBI had been pre-treated with the reducing agent dithiothreitol (DTT), so that all disulfide bridges in the BBI were cleaved.

In the preparation indicated by "BBI-A," the BBI was first reduced and then the cysteine residues were alkylated with iodoacetamide, to avoid a re-oxidation of the cysteine residues. For this reduction, 8 mg BBI in 0.5 ml PBS and 0.2 ml denaturing buffer (12.5 mM Tris pH 6.8, 80 µl EDTA, 1% SDS and 20% glycerol) in 20 µl fresh 2.6 M DTT solution were boiled for 10 min. The alkylation took place after the reduction by addition of 70 µl 20% iodoacetamide solution for 60 minutes at 20°C. The preparation were then dialyzed four times against PBS for 24 hours.

The four preparations 0, BBI, BBI-R, and BBI-A were either not treated with ionizing radiation (0 Gy) or irradiated with a single 2-Gy dose.

This test was repeated in several independent experiments and the respective results were averaged. The error bars give the dispersion of the values.

As is to be seen from Fig. 1, the addition of BBI, reduced BBI or reduced and alkylated BBI without irradiation (0 Gy) does not influence the survival of the cells within the limits of measurement accuracy. Without the addition of BBI (0), the clonogenic survival of human dermal fibroblasts after irradiation with a single dose of 2 Gy is reduced by ca. 30 to 40%.

A 16-hour pre-treatment with BBI (BBI), reduced BBI (BBI-R) or reduced and alkylated BBI (BBI-A) led to a significant increase in the clonogenic survival of the irradiated human dermal fibroblasts by 20 - 30%.

Significant differences between BBI, BBI-R and BBI-A could not be detected within the limits of measurement accuracy

The result of the clonogenic assay shown in Fig. 1 verifies that modified forms of BBI .e. its reduced form or reduced and alkylated form, display just as high a radio-protective effect as the unaltered BBI.

B. Inhibitor effect

It was next studied whether the modified forms of BBI act as protease inhibitors towards chymotrypsin or not. For this, a protease inhibition test was carried out that is explained briefly below.

1.3. Protease inhibition test

A preparation with 50 µl TLCK-treated chymotrypsin (0.1 mg/ml) was incubated for 10 minutes with 50 µl of a solution with BBI (BBI), reduced BBI (BBI-R), or reduced and alkylated BBI (BBI-A) together with 50 µl PBS and 50 µl of the chymotrypsin substrate acetyl-A-A-P-F-pNa (0.5 mg/ml from Bachem, Heidelberg, FRG) in 12.5% DMSO, 87.5% PBS. The colored reaction product is detected at 405 nm in a spectrophotometer.

If the inhibition of the protease trypsin (see Example 4, Fig. 8) is determined, the peptide CBZ-R-pNa (1 mg/ml) and also TPCK-treated trypsin (0.1 mg/ml) is used as the substrate and the test is otherwise carried out as described.

A preparation without BBI (0) was carried out as a control, and its measurement value was compared with 100% chymotrypsin activity (% CH). The blank with buffer alone is indicated by "-" and gives the background of the buffer solution and the cell.

1.4. Inhibition of chymotrypsin by BBI and modified BBI forms

The result of the chymotrypsin test is shown in Fig. 2. While the whole BBI inhibits the chymotrypsin activity by 80%, the reduced form of the BBI (BBI-R) inhibits the chymotrypsin activity by only ca. 30%. On the other hand, the reduced and alkylated form of the BBI (BBI-A) is no longer in a position to inhibit the chymotrypsin activity.

The undesirable side effect on use of the modified forms of BBI (BBI-R and BBI-A) as radio-protective agents, that chymotrypsin, a digestive enzyme, is inhibited at the same time, is thus greatly reduced or is completely absent.

Example 2. Cleavage of the BBI with cyanogen bromide and pepsin, and the analysis of the cleavage products for protease inhibition and radio-protective activity

In this experiment it was demonstrated that part fragments of BBI also display a radio-protective effect.

2.1. Cleavage of the BBI

The whole BBI was digested with cyanogen bromide (CNBr) and with the gastric enzyme pepsin. For this, 50 mg BBI were diluted in 1.5 ml 70% formic acid. Then 118 mg cyanogen bromide were added and the preparation was incubated for 20 hours at 4°C. The reaction mixture was diluted with water and lyophilized. The lyophilizate was then digested with 340 U pepsin at pH 2.5 for 24 hours at 40°C. To end the digestion reaction, formic acid was added.

2.2. Separation of cleavage products

The cleaved material was then separated by molecular sieve chromatography on a Sephadex G25 column. During the column run, 110 fractions were obtained, the inhibiting effect against trypsin or chymotrypsin of which was investigated in the protease inhibition test shown under 1.3.

2.3. Protease inhibition

Fig. 3 shows the result of the protease inhibition of the individual column fractions, with the round dot symbol representing the trypsin-inhibiting activity (anti-trypsin) and the lozenge-shaped symbol the chymotrypsin-inhibiting activity (anti-chymotrypsin).

Altogether three peaks are shown: one peak (F1) at fractions 30-50, in which both trypsin-inhibiting activity and also chymotrypsin-inhibiting activity could be detected. Another peak (F2) at fraction 60, in which chymotrypsin-inhibiting activity was detectable, and a third peak (F3) at fraction 72-73, in which chymotrypsin-inhibiting [sic; should be trypsin-inhibiting, see next paragraph and Fig. 3] activity could be detected.

The fractions included in the first peak (F1) contain mainly the uncleaved total-BBI, which inhibits both trypsin and chymotrypsin. The fractions marked F2 and F3 contain two cleavage products of the BBI, one being the cleavage product with chymotrypsin-inhibiting activity (F2) and the other the cleavage product with trypsin-inhibiting activity (F3).

2.4. Clonogenic assay

The three fractions, F1, F2, F3 were used in a clonogenic assay, as was described under 1.1. The dermal fibroblasts were treated with the fractions F1 to F3 before irradiation with a single dose of 2 Gy for 16 hours, and their clonogenic survival in comparison to irradiated control cells (0) without addition of a BBI product was analyzed.

All three fractions, according to Fig. 4, display a distinct rise in the clonogenic survival, with fraction F2, the cleavage product with chymotrypsin-inhibiting activity, displaying a considerably higher radio-protective effect than uncleaved whole BBI (F1) or the cleavage product with trypsin-inhibiting effect (F3).

Through the results shown here it is demonstrated that peptide fragments of the BBI produced by chemical and enzymatic cleavage include just as high or even a considerably higher radio-protective effect than the whole BBI.

Example 3. Radio-protective effect of the nona- and heptapeptides in accordance with the invention

In another clonogenic assay (see 1.1.), the radio-protective effect of a total of four BBI fragments in accordance with the invention were studied in comparison to the whole BBI.

In the test, whole BBI (BBI) was used, as well as four peptides, chemically synthesized by the Merrifield synthesis, whose C-terminal amino acids had acetyl groups as protective groups, and whose N-terminal amino acids had amide groups. The sequences of these peptides are given in the attached Sequence Protocol.

The peptide P1 displays the sequence SEQ ID No.: 1, with its terminal cysteine residues being linked by way of a disulfide bridge, so that the peptide P1 has a cyclic structure. The peptide P2 has the sequence SEQ ID No.: 3. It contains no cysteine residues and therefore takes on a linear conformation.

The peptide P3 corresponds to the peptide P1, thus has the sequence SEQ ID No 1, but with its terminal cysteine residues not linked by way of a disulfide bridge.

The peptide P3 thus, like P2, has a linear structure.

The peptide P4 has the sequence SEQ ID No.: 2 and thus displays a serine → valine amino acid exchange, relative to the peptides P1 and P3 that have the sequence SEQ ID NO.: 1. Its terminal cysteine residues are not linked by way of a disulfide bridge, so that the peptide P4 also has a linear structure.

The peptides P1, P3 and P4 are nonapeptides with nine amino acids, but the peptide P2 is a heptapeptide with only seven amino acids.

In Fig. 5, the result of the clonogenic assay is shown. A change relative to the method described under 1.1. consists in that the irradiation of the fibroblasts was carried out with a single dose of 4 Gy.

Besides the preparations in which whole BBI or the peptides P1 were added, two control preparations were also carried out: one a preparation with unirradiated fibroblasts without addition of BBI or a peptide (U), the other a preparation with irradiated fibroblasts, but likewise without the addition of BBI or one of the peptides (0).

The whole BBI was used at a concentration of 10 µM, the peptides P1 - P4 at a concentration of 80 µM in each case.

As can be seen from Fig. 5, all the peptides display a radio-protective effect that is comparable to that of the whole BBI. The peptides P1 and P3, i.e. the linear and cyclic form of the sequence SEQ ID No.: 1, were more pronounced in their radio-protective effect compared with the peptide P2 (heptapeptide) and P4 (base exchange).

The peptides in accordance with the invention thus display for human dermal fibroblasts a protective effect against ionizing radiation that is comparable to the whole BBI which is eight to ten times larger.

It is shown in Fig. 6 that the peptides exert a protective effect, not only against ionizing radiation but also against UV radiation, that is as high or higher than that of the whole BBI.

A clonogenic assay was again carried out, in which the whole BBI or the four peptides P1 to P4 were used.

Here, the radiation took place with UV-B radiation of 100 joules/m^2

The clonogenic survival of the human dermal fibroblasts was elevated by about 40% on addition of whole BBI compared with the untreated cells. The addition of the peptide P1 had just as pronounced an effect as that of the addition of whole BBI, and the addition of the peptides P2 and P3 showed an even more pronounced effect that was another 20% higher than that of the whole BBI.

The fragment P4 which, compared with the peptide P3 displays a base-exchange of serine→valine, shows compared with the whole BBI a lesser effect on the clonogenic survival of the fibroblasts. In comparison to the controls (0), however, the addition of P4 raises the clonogenic survival of the dermal fibroblasts significantly.

The experiments shown in Figs. 5 and 6 verify that the peptides in accordance with the invention exhibit a radio-protective effect both against ionizing radiation and also against UV radiation that is comparable to or sometimes even better than that of the whole BBI.

Thus the peptides in accordance with the invention are ideally usable as radio-protective agents.

Example 4. Protease-inhibiting activity of the peptides in accordance with the invention

In the experiments with the results shown in Figs. 7 and 8, the protease-inhibiting activity of BBI was compared with that of the peptide fragments P1 to P4, that were already presented in Example 3.

The protease inhibition test was carried out as described under 1.3. The inhibition of chymotrypsin is shown in Fig. 7, and the inhibition of trypsin in Fig. 8.

The control, indicated by 0, shows the chymotrypsin or trypsin activity in the absence of BBI or a peptide in accordance with the invention.

As can be seen from Fig. 7, an addition of 0.05 mM and 0.1 mM whole BBI (BBI) inhibits the chymotrypsin activity by 80 or 90%. In comparison to this, the addition of 0.1 mM of the peptide P1, P2, P3, or P4 inhibits the chymotrypsin activity either not at all or only by a few percent.

As is seen from the results shown in Fig. 8, the same holds for the trypsin-inhibiting activity. While the whole BBI in a concentration of 0.05 mM or 0.1 mM inhibits the activity of trypsin by over 90%, the addition of 0.1 mM of each of the peptides P1, P2, P3, or P4 has no effect on the activity of trypsin.

This example shows that none of the peptides in accordance with the invention exert an inhibitory effect on the proteases chymotrypsin or trypsin.

The peptides in accordance with the invention with radio-protective effect therefore do not have the undesired side effect of at the same time blocking the digestive enzymes chymotrypsin and trypsin. As was shown in experiments with rats, the blocking by protease inhibitors of these proteases produced in the pancreas leads to severe damage to the pancreas.

The peptides in accordance with the invention are thus suitable for use for the radio-protection of humans, the more so since the peptides P1 to P4, because of their small size, diffuse rapidly and can therefore be distributed well. Because of the small size of the peptides, immunological reactions are also not a consideration.

An especially good effect is exhibited by the peptides P1 to P4 in the protection against UV radiation, as was shown in embodiment example 3 in connection with Fig. 6. Since the peptides containing only nine or seven amino acids can also penetrate into the skin, they are particularly well suited for the protection of human skin against elevated sun exposure.

SEQUENCE PROTOCOL (5402P148WO)

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Eberhard-Karls-Universitaet Tuebingen;
University Clinic
(B) STREET: Geissweg 3

(C) PLACE: Tuebingen
(E) COUNTRY: DE
(F) ZIP CODE: D-72076

(ii) NAME OF INVENTION: Peptide with radio-protective effect

(iii) NUMBER OF SEQUENCES: 3

(iv) COMPUTER-READABLE VERSION:
(A) DATA CARRIER: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPA)

(2) INFORMATION ON SEQ ID No.: 1

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 Amino acids
(B) TYPE: amino acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: linear or cyclic

(ii) TYPE OF MOLECULE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(xi) DESCRIPTION OF SEQUENCE: SEQ ID No.: 1

Cys Ala Leu Ser Tyr Pro Ala Gln Cys
1 5

(2) INFORMATION ON SEQ ID No.: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 Amino acids
(B) TYPE: amino acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: linear or cyclic

(ii) TYPE OF MOLECULE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(xi) DESCRIPTION OF SEQUENCE: SEQ ID No.: 2

Cys Ala Leu Val Tyr Pro Ala Gln Cys
1 5

(2) INFORMATION ON SEQ ID No.: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 Amino acids
(B) TYPE: amino acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: linear or cyclic

(ii) TYPE OF MOLECULE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(xi) DESCRIPTION OF SEQUENCE: SEQ ID No.: 3

Ala Leu Ser Tyr Pro Ala Gln
1 5

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Patent Claims

1. Peptide with radio-protective effect, characterized by the fact that it includes a modified form and/or if necessary a modified fragment of the Bowman-Birk protease inhibitor (BBI).

2. Peptide as in claim 1, characterized by the fact that it displays no protease-inhibiting effect against trypsin and chymotrypsin.

3. Peptide as in claims 1 or 2, characterized by the fact that it has at least two cysteine residues that are present in reduced form.

4. Peptide as in one of the claims 1 to 3, characterized by the fact that at least some of its amino acid residues are present in alkylated form.

5. Peptide as in one of the claims 1 to 4, characterized by the fact that it has less than 20 and preferably fewer than 10 amino acids.

6. Peptide as in claim 5 with the sequence SEQ ID No.: 1

Cys Ala Leu Ser Tyr Pro Ala Gln Cys

7. Peptide as in claim 5 with the sequence SEQ ID No.: 2

Cys Ala Leu Val Tyr Pro Ala Gln Cys

8. Peptide as in claim 6 or 7, characterized by the fact that its terminal cysteine residues are bound covalently to each other.

9. Peptide as in claim 5 with the sequence SEQ ID No.: 3:

Ala Leu Ser Tyr Pro Ala Gln

10. Peptide as in one of the claims 1 to 9, characterized by the fact that at least one of its amino acids has a protective group.

11. Peptide as in claim 10, characterized by the fact that its C-terminal amino acid displays an acetyl group.

12. Peptide as in claim 10 or 11, characterized by the fact that its N-terminal amino acid displays an amide group.

13. Use of one or more of the peptides as in one or more of the claims 1 to 12 as a radio-protective agent.

14. Use as in claim 13 for protection against ionizing radiation, in particular of normal tissue in the radiation therapy of tumor patients.

15. Use as in claim 13 for the protection of the skin against UV radiation, in particular against the UV radiation in sunlight

16. Pharmaceutical composition, in particular for intravenous administration, characterized by the fact that it contains one or more of the peptides in accordance with one or more of the claims 1 to 12 in a radio-protectively efficacious amount.

17. Cosmetic composition for application to the skin, characterized by the fact that it contains one or more of the peptides in accordance with one or more of the claims 1 to 12 in a radio-protectively efficacious amount.

18. Nucleic acid that displays a sequence section coding for a peptide as in one of the claims 1 to 10 as well as if necessary control sequences necessary for the expression of the nucleic acid.

19. Nucleic acid as in claim 18, characterized by the fact that it is contained in an expression vector.

20. Method for production of a peptide in accordance with one of the claims 1 to 10, characterized by the fact that a nucleic acid as in claim 18 or 19 is expressed using the methods of molecular biology.

21. Method for production of a peptide in accordance with one of the claims 1 to 10, characterized by the fact that the unmodified Bowman-Birk protease inhibitor is cleaved proteolytically and/or chemically.

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Fig. 1, 2, 3 [Fraktion = fraction], 4, 5, 6, 7, 8

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INTERNATIONAL SEARCH REPORT International File No.
PCT/EP98/04051

Field 1. Comments on the claims that have been shown to be unsearchable (Continuation of item 1 on page 1)

According to Article 17(2)a) no search report was provided for certain claims on the following grounds:

1. Claim No.
Because you are referring to subjects that the authorities are not obligated to search, namely:

Comment: Although the claims 13-15 relate at least in part to a method for the treatment of the human/animal body, the search was carried out and was based on the stated effects of the compound/composition.

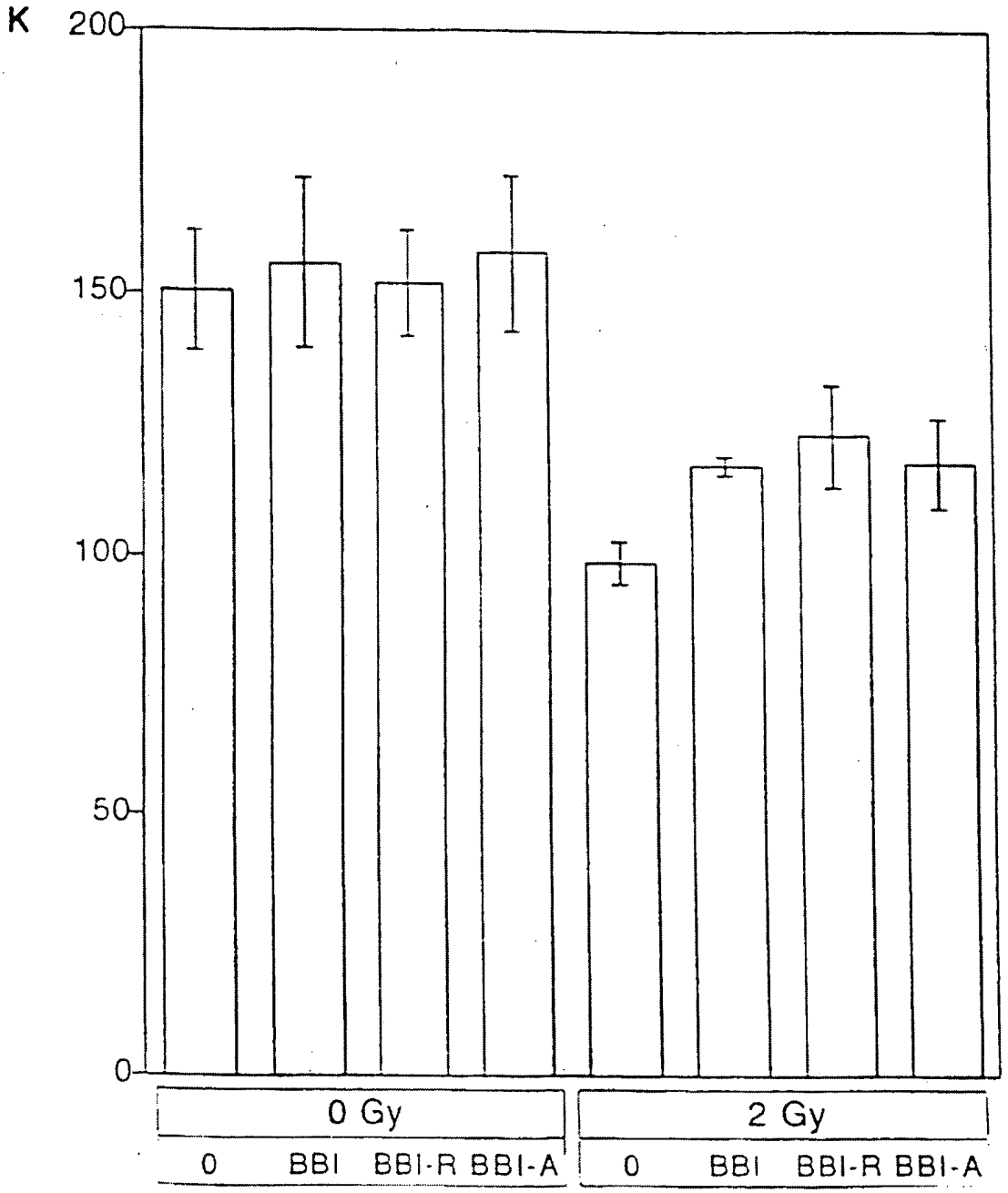


Fig. 1

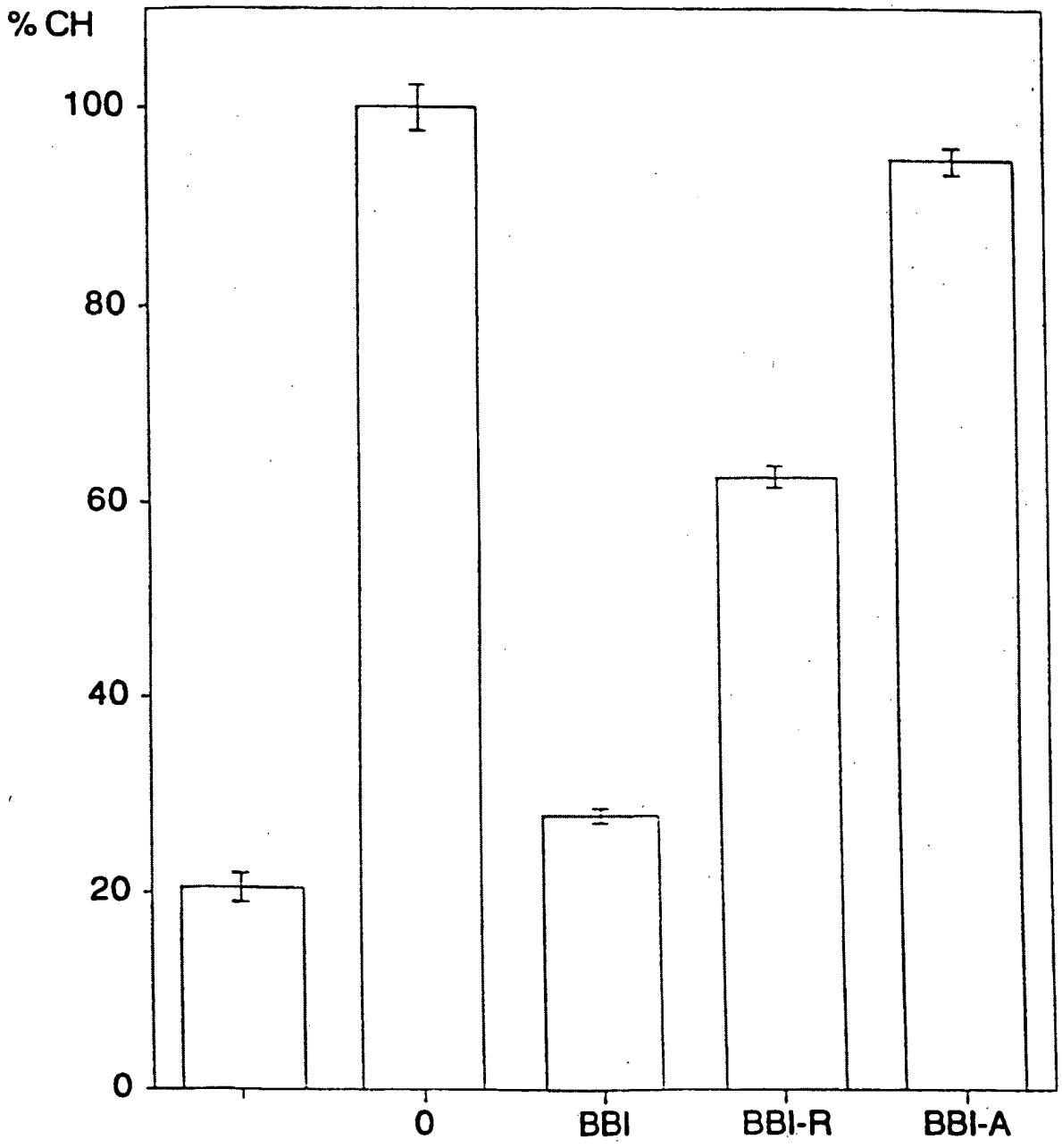
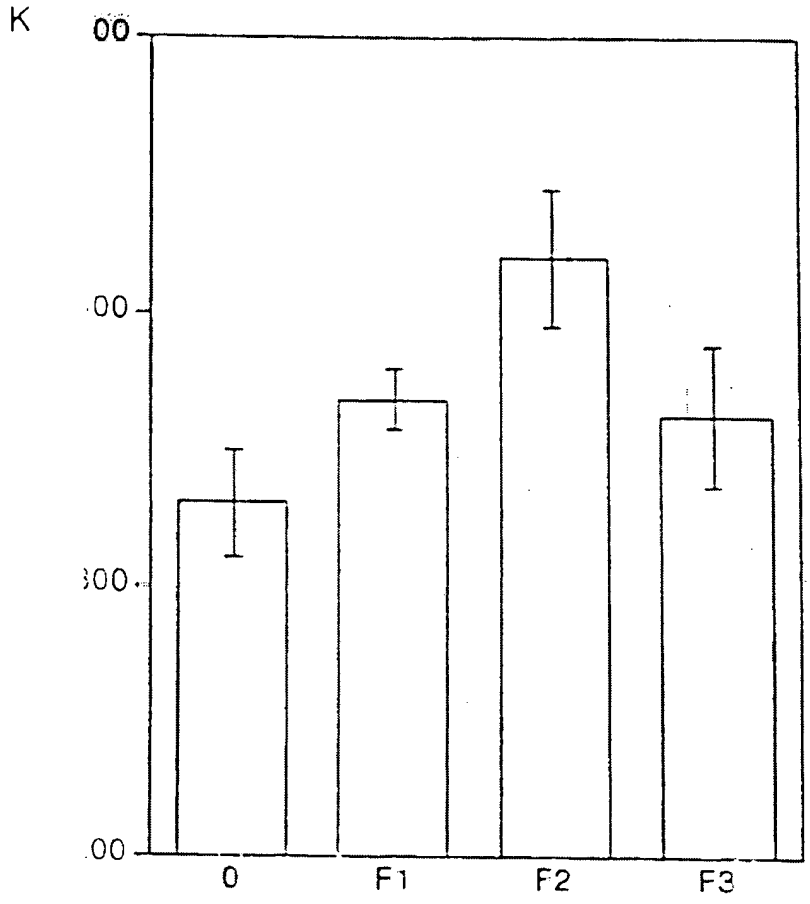


Fig. 2



Fg 4

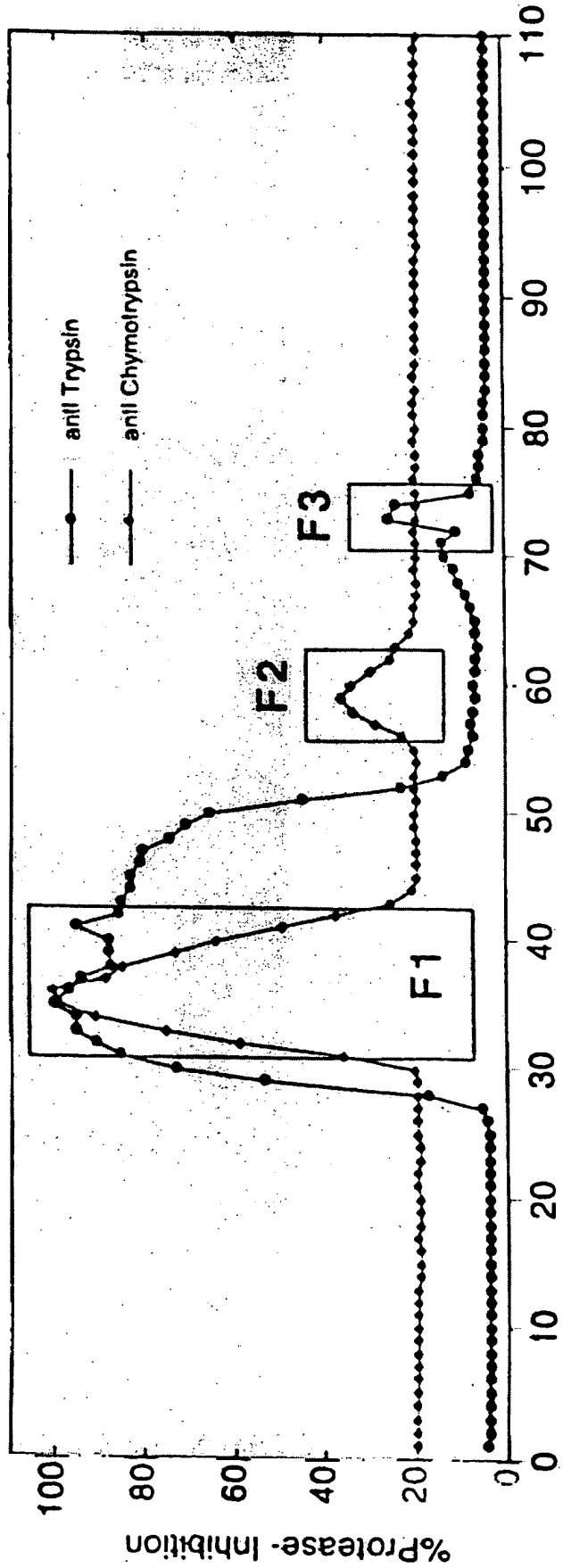

%Protease- Inhibition
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
90
100
110
Fraktion
Fraction
anti Trypsin
anti Chymotrypsin
F1
F2
F3

Fig. 3

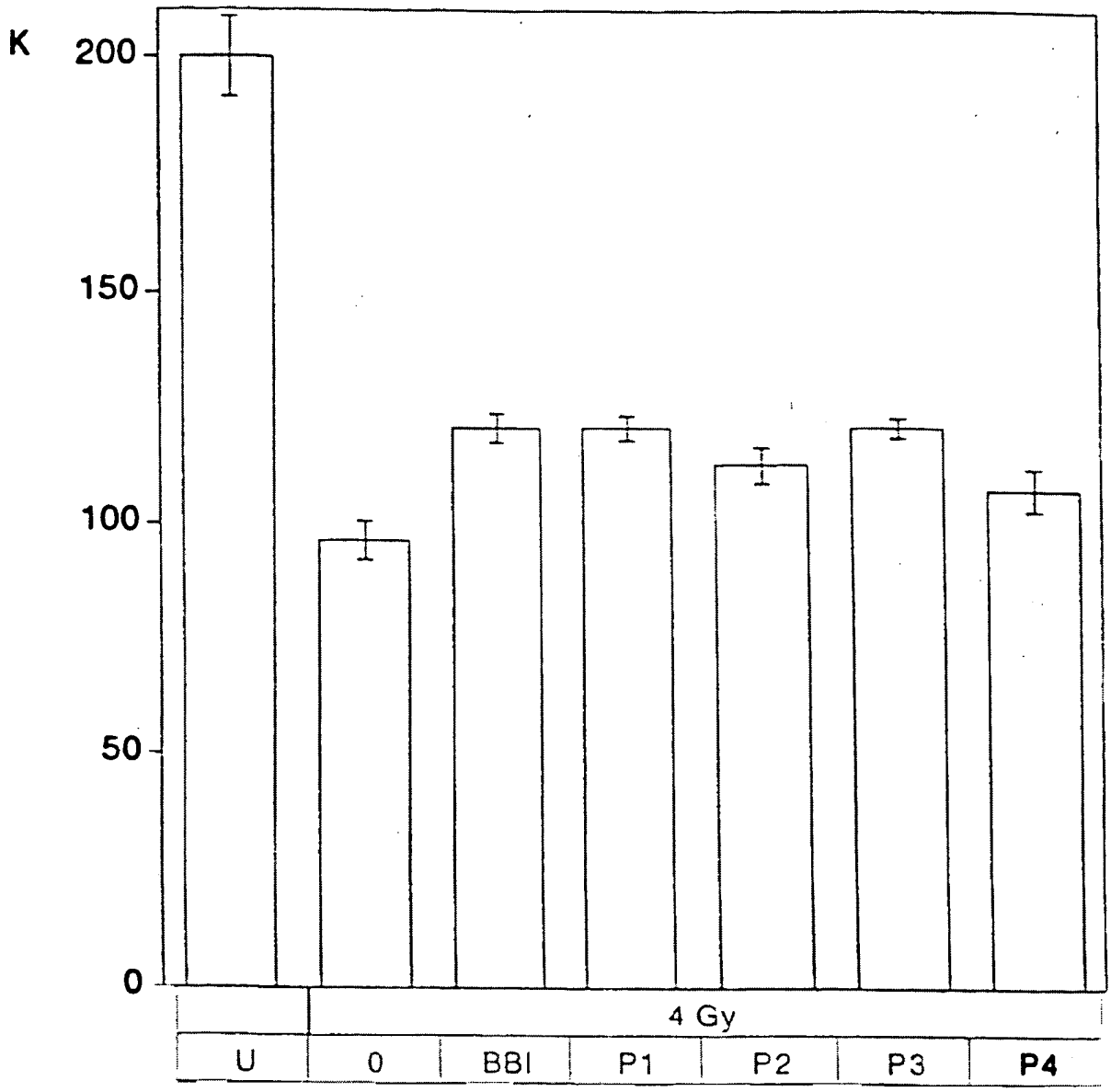


Fig. 5

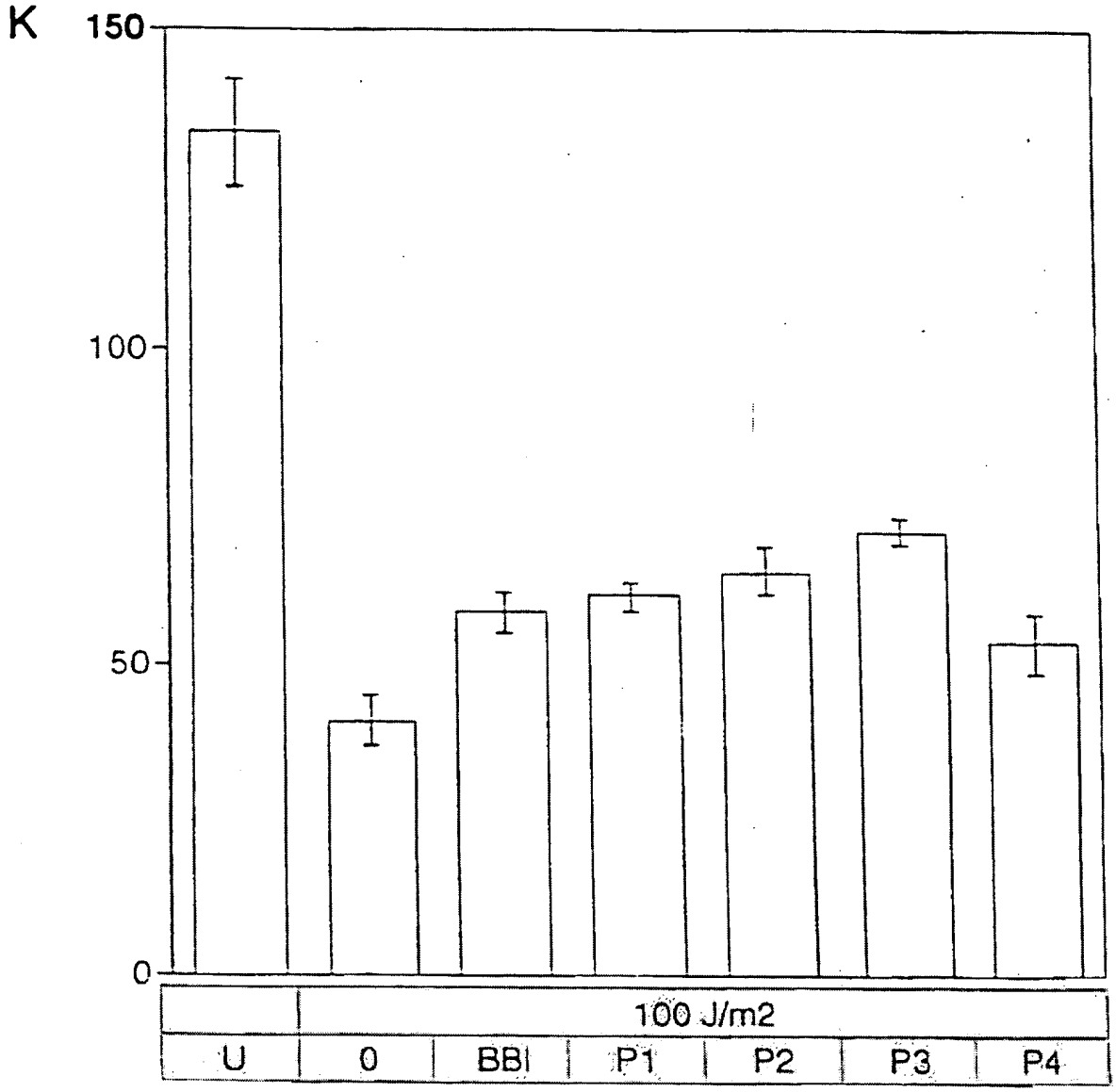


Fig. 6

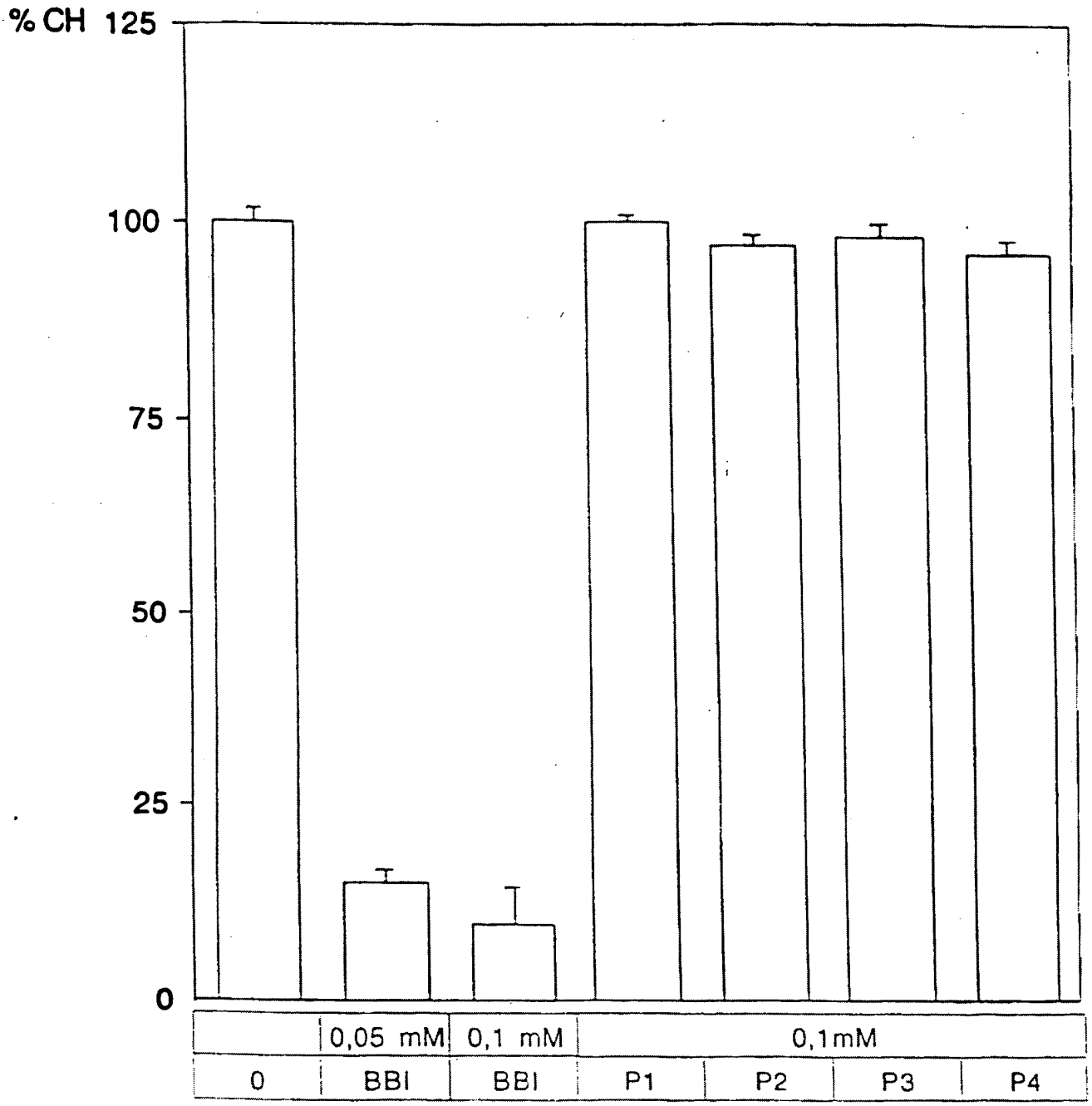


Fig. 7

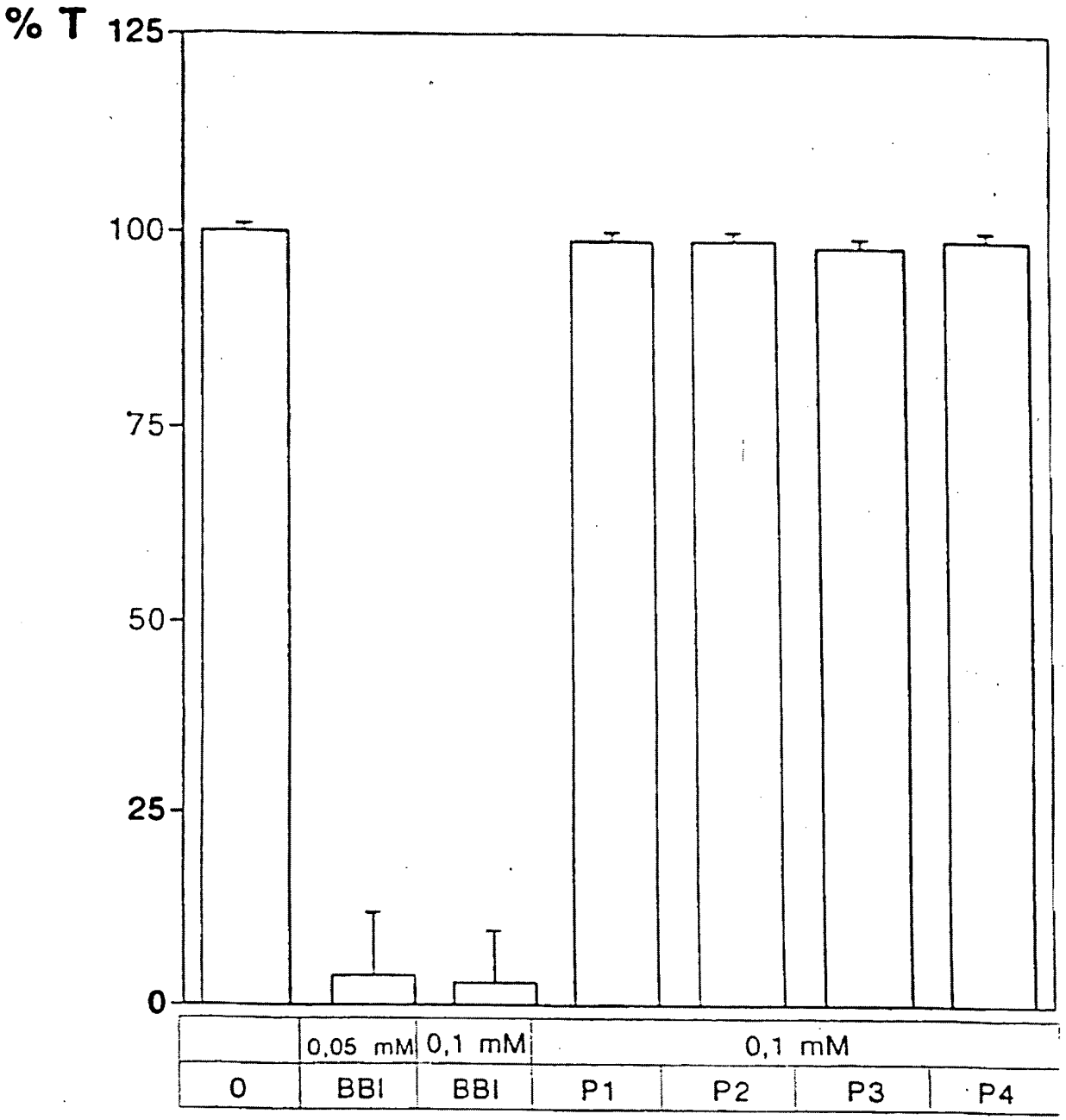


Fig. 8

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/EP 98/04051

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/81 A61K38/55 A61K7/40

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
Y	WO 91 07166 A (UNIV WASHINGTON) 30 May 1991 see examples 1-4	1-21
Y	WO 94 20121 A (UNIV PENNSYLVANIA ;CENTRAL SOYA CO (US)) 15 September 1994 see abstract	1-21
Y	WO 94 09802 A (UNIV PENNSYLVANIA ;CENTRAL SOYA CO (US)) 11 May 1994 see abstract	1-21

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 November 1998	01/12/1998

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk

Authorized officer

INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/EP 98/04051

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No
Y	S. ANDO ET AL: "Anti-chymotrypsin and anti-elastase activities of a synthetic bicyclic fragment containing a chymotrypsin-reactive site of soybean Bowman-Birk inhibitor" BIOCHIMICA ET BIOPHYSICA ACTA. vol. 916, 1987, pages 527-531, XP002083657 see abstract see table I see page 530, column 2, last paragraph	1-21
Y	S. TERADA ET AL: "Studies on the synthesis of proteinase inhibitors" INTERNATIONAL JOURNAL OF PEPTIDE AND PROTEIN RESEARCH. vol. 15, 1980, pages 441-454, XP002083658 COPENHAGEN DK see table I	1-21
Y	M. WAKI ET AL: "synthesis and inhibitory properties of reactive-site peptides of protease inhibitors from peanuts and cucumber" PEPTIDE CHEMISTRY,1987, pages 657-662, XP002083659 see table I	1-21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 98/04051

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

[X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Observation: Although Claims 13-15 relate at least partially to a method for treatment of the human or animal body, the search was carried out and was based on the cited effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

.nformation on patent family members

Inter nal Application No
PCT/EP 98/04051

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9107166	A	30-05-1991	NONE		
WO 9420121	A	15-09-1994	US	5376373 A	27-12-1994
			AU	674403 B	19-12-1996
			AU	6395994 A	26-09-1994
			EP	0692967 A	24-01-1996
			NO	953556 A	31-10-1995
WO 9409802	A	11-05-1994	US	5338547 A	16-08-1994
			AU	5597594 A	24-05-1994
			EP	0695188 A	07-02-1996
			US	5376373 A	27-12-1994